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# Auxin-Induced Epinasty of Tobacco Leaf Tissues[1]

## A Nonethylene-Mediated Response

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**Interveinal strips (10 × 1.5 mm) excised from growing tobacco (*Nicotiana tabacum* L. cv Xanthi) leaves curled >300° when incubated for 20 h in 5 to 500 μM α-naphthalene acetic acid or 50 to 500 μM indole-3-acetic acid. Epinasty was not induced without auxin or by the auxin analog β-naphthalene acetic acid, and less substantial epinasty was induced in midrib and vein segments. Auxin treatment increased the length of both surfaces of strips. Curvature resulted from greater growth on the adaxial side. Epinastic sensitivity of strips to auxin appeared first in the distal third of young leaves (blade 4.5–6.0 cm). In older leaves (8–10 and 12–14 cm), the interveinal tissues throughout were sensitive, whereas in leaves 16- to 18-cm long, sensitivity was reduced in the distal two-thirds. Amino-oxyacetic acid (AOA), an ethylene biosynthesis inhibitor, partially inhibited epinasty at 100 μM. However, a poor correlation between inhibition of ethylene biosynthesis by AOA and its inhibition of curvature and the inability of ethylene to produce epinasty or to reverse the effects of AOA suggests that auxin-induced epinasty is not caused by auxin-induced ethylene production.**

Auxin has long been thought to have only a limited role in the growth of leaves. Avery (1935) first reported that treatment of young tobacco (*Nicotiana tabacum* L.) leaves with auxin produced epinasty (dorsoconvex curvature) resulting from growth within the midrib and that auxin was without effect on the lateral veins and interveinal tissues. Went and Thimman (1937) also detected auxin-induced elongation growth of the midrib and the lateral veins and confirmed that auxin treatment failed to increase the surface of the mesophyll. In summarizing the early auxin investigations, these authors also concluded that all of the available data "fit in with the view that auxin causes elongation of leaf veins, while the growth of the mesophyll depends on other factors" (Went and Thimman, 1937). This idea that leaf development is controlled by chemically distinct vein growth factors or "caulocaline" and mesophyll growth factors or "phyllocaline" (Went, 1951) was supported by the observation that treatment with adenine (Bonner and Haagen-Smit, 1939) and cytokinins (Engelke et al., 1973) promoted mesophyll growth but not veinal growth.

Auxin is now known to induce ethylene production (Abeles et al., 1992). Ethylene treatment is known to result in epinasty in about one-third of the species tested, although the curvature is generally limited to the leaf petioles (Abeles et al., 1992). Several varieties of tobacco are not epinastic in response to carbon monoxide (Zimmerman et al., 1933), an ethylene analog (Abeles et al., 1992).

A larger role for auxin in leaf growth, however, is suggested by some recent investigations. A transient auxin-induced hyponasty (dorsoconcave curvature) response by growing leaves from bean (*Phaseolus vulgaris*) was described (Lippincott and Lippincott, 1971; Hayes and Lippincott, 1976; Hayes, 1977). Both veinal and interveinal tissues appeared to contribute to this response, which apparently does not result from auxin induction of ethylene production (Hayes, 1981). Another study (Santoni et al., 1990, 1991; Masson et al., 1996) showed that ATPase and proton-pumping activities of isolated and purified plasma membranes from tobacco leaves are stimulated by auxin. A third study indicated that the membrane potential of protoplasts prepared from tobacco leaf mesophyll is also sensitive to auxin (Ephritikhine et al., 1987; Barbier-Brygoo et al., 1989, 1991; Venis et al., 1990, 1992).

In light of these recent studies, we chose to test whether auxins would induce the growth of excised tobacco leaf tissues and whether the reported results from the tobacco-leaf system might therefore reflect aspects of a generalized auxin-induced growth response in leaves. Preliminary overnight experiments showed that auxin strongly induced epinasty of leaf discs from the interveinal regions of young tobacco leaves. We further characterized this response using 1-cm-long strips of leaf tissues with which epinastic curvature could be more easily quantified.

## MATERIALS AND METHODS

Approximately 200 tobacco (*Nicotiana tabacum* L. cv Xanthi) plants were grown individually in 10- × 10-cm pots in a soil mixture containing 67% (v/v) peat moss and 33% pumice supplemented with $KNO_3$ (0.6 g $L^{-1}$), Treble Superphosphate 0–45-0 (0.7 g $L^{-1}$), limestone flour (4.1 g $L^{-1}$), dolomite (4.1 g $L^{-1}$), $FeSO_4$ (0.6 g $L^{-1}$), and Nitroform (Nor-Am Chemical, Wilmington, DE) 38-0-0 (0.9 g $L^{-1}$).

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Abbreviations: AOA, amino-oxyacetic acid; Btp, 1,3-bis(tris[hydroxymethyl]methylamino)propane; 1-NAA, α-naphthalene acetic acid; 2-NAA, β-naphthalene acetic acid.

Daily watering was with Peters Peat Lite Special 20-10-20 (W.R. Grace, Tukwila, WA) diluted to 100 $\mu$M N and supplemented with 5 $\mu$M Fe from iron chelate dispersable powder (Miller Chemical and Fertilizer, Hanover, PA) and Mg at 10 $\mu$M from $MgSO_4$ or with Peters Dark Weather Fertilizer 15-0-15 (W.R. Grace), also diluted to 100 $\mu$M N and supplemented with Fe as above and with 2.5 $\mu$M Si from $NaSiO_3$ or (once a week) with water alone. Plants were grown under greenhouse conditions with the temperature controlled between 18 and 23°C (day) and between 13 and 16°C (night). Supplemental lighting from metal halide lamps maintained a minimum 17-h daylength. To provide a continuous supply of growing leaves, the plants were cut back to within a few centimeters of the roots every 6 weeks and allowed to regrow at least 3 weeks before experimental use.

### Curvature Assays

Growing leaves 8 to 10 cm in length (except where indicated) were harvested from plants with at least six mature leaves. All subsequent manipulations were conducted in dim green light (1.2 $\mu$mol $m^{-2}$ $s^{-1}$) at 25°C. Strips of interveinal leaf tissues 10-mm long and 1.5-mm wide were cut from the leaves using double-bladed cutters. Except where indicated, the interveinal strips were cut parallel to the lateral or secondary veins radiating from the midrib. Strips were cut from the distal two-thirds of the leaf (except in one experiment, in which strips were prepared separately from each third of the leaf [base, middle, and tip] from leaves of various lengths [ages]). Each leaf typically yielded between six and eight strips (Fig. 1), which were distributed between different treatments. In one experiment, the tissues to either side of leaf midribs were trimmed away to within 0.5 mm, and then 10-mm-long sections were cut from the middle one-third of the isolated midribs. Secondary vein sections were prepared in a similar fashion. In most experiments the strips were placed immediately into 60- × 15-mm Petri dishes containing 10 mL of a control solution consisting of 10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp (pH 6.0), or a solution also including auxin or AOA as indicated. Incubations were conducted in extremely dim green light (<0.01 $\mu$mol $m^{-2}$ $s^{-1}$) at 25°C. For experiments to measure the evolution of ethylene and those testing the effect of ethylene treatment, incubation was in 25-mL Erlenmeyer flasks containing 5 mL of solution and closed with serum stoppers.

One millimolar 1-NAA, 2-NAA, and IAA stock solutions were prepared by dissolving in 100 $\mu$L of ethanol, diluting with 50 mL of equimolar control solution (as described above but also containing 1 mM KOH), and heating briefly to 80°C. An AOA stock solution (1 mM) was prepared by dissolving in control solution, after which the pH was readjusted to 6.0 by the addition of Btp. For ethylene treatment, an aliquot of air was removed from the Erlenmeyer flask and replaced by an ethylene-containing injection (Scott Specialty Gases, Plumsteadville, PA) following enclosure of strips and solution.

Strips and vein segments were incubated for 20 h in most experiments, after which they were gently removed from solution and placed on the stage of a dissecting microscope where the curvature of each was assessed. Curvature was determined by viewing each strip from the side (as illustrated in Fig. 1B) against a protractor. The angle created by the interception of the tangent to the two terminal portions of the strip was measured and recorded in degrees.

All experiments were performed three times with similar results, except for those experiments described as limited, which were performed only once or twice. All error bars indicate the 95% confidence limit.

### Elongation Measurement

In one experiment, estimates of the changing length of the adaxial (dorsal) surface of interveinal strips treated with and without auxin (10 $\mu$M 1-NAA) for 20 h were made by digitizing images of strips viewed from the side (as in Fig. 1B) using a dissecting scope attached to a digitizing camera (model CCD-72SX, Dage-MTI, Michigan City, IN) and using NIH Image 1.41 software. The length of the curved surfaces was then determined by tracing the outline of strips in the digitized images using a digitizing tablet (Kurta, Phoenix, AZ) and SigmaScan software (Jandel Scientific, Corte Madera, CA).

For the measurement of palisade mesophyll cell enlargement, two interveinal strips were prepared from a single 8- to 10-cm-long leaf: one strip from each side of the leaf from the same point approximately 3 cm from the distal end. One strip was then treated with auxin (10 $\mu$M 1-NAA) for 20 h and the other was treated without auxin as in other experiments. Each strip was then vacuum-infiltrated with water to prevent refraction, cut into 1- to 2-mm lengths, placed adaxial side up on glass slides, and covered, but not flattened, by a glass coverslip. The palisade layer of the mesophyll within segments of the strips was then viewed through a light microscope at ×200 under conditions of minimum depth of field. Transects 0.4-mm-long (six per interveinal strip) were then established using an eyepiece micrometer. The transects were placed to avoid gaps in the palisade layer at substomatal cavities but in other respects were located randomly. The number of cells intercepted by the transects were then counted as a relative measure of mesophyll cell size between the two treatments. This experiment was performed four times and the data were pooled.

### Ethylene Measurement

Gas samples (0.6 mL) were removed by syringe from the serum-stoppered Erlenmeyer flasks for gas chromatograph injection. Ethylene was detected using a gas chromatograph (model GC-mini 2, Shimadzu, Columbia, MD) equipped with a flame ionization detector and a 6 × 1/8-inch (15.2 cm × 3.2 mm) stainless steel column packed with Porapac N 80/100 (Alltech, Deerfield, IL). The oven temperature was 60°C and the detector was 80°C with gas flow rates of 0.5, 1.0, and 1.5 kg $cm^{-2}$, respectively, for air (oxygen), hydrogen, and nitrogen (carrier). Concentration was computed with standard curves prepared with ethyl-

ene standards from Scott Specialty Gases (Plumsteadville, PA).

## RESULTS

Interveinal strips were cut from the leaves as shown in Figure 1. Strips (Fig. 1B, no. 2) typically displayed a small degree of epinastic curvature (30.0 ± 5.8° [95% confidence limit]; $n = 20$) immediately after preparation. Incubation in the control solution (10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp [pH 6.0]) for 20 h (Fig. 1B, no. 3) increased the length of the strips (8.5 ± 1.4% of initial; $n = 12$) but did not significantly change their curvature (i.e. 37.2 ± 15.2°; $n = 20$). Incubation in auxin (10 μM 1-NAA), however, dramatically increased curvature (Fig. 1B, no. 4). The curvature induced by auxin in the interveinal strips was always epinastic. This was readily evident because the abaxial (ventral) surface had a slightly whitish cast due to the presence of subepidermal air spaces and because any vasculature included in the strips was evident protruding from the abaxial side.

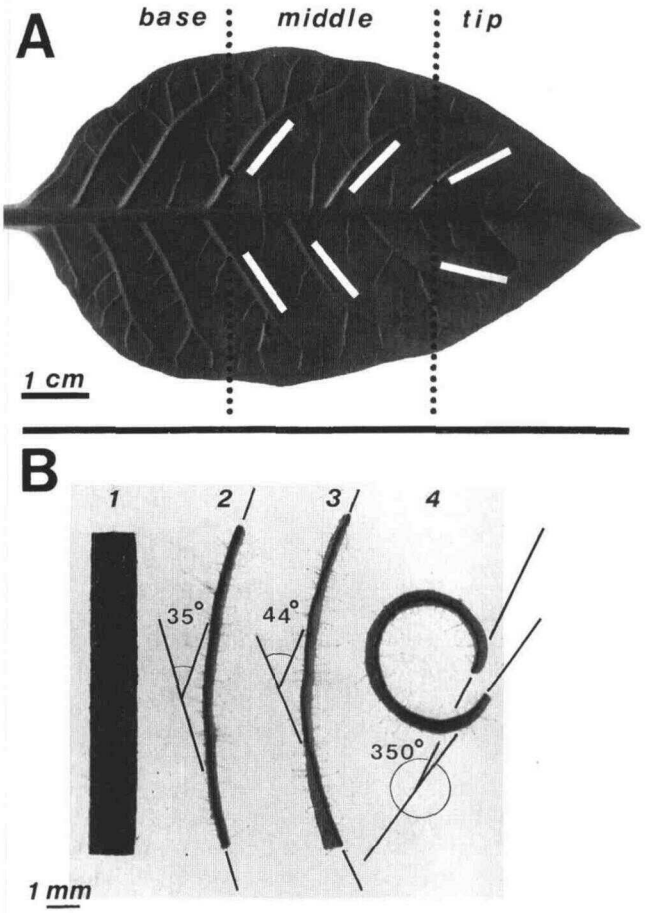


**Figure 1.** Young tobacco leaf viewed from the abaxial side illustrating the sites of interveinal leaf strip excision (A) and an example of auxin-induced epinasty of leaf strips, together with an illustration of the method of strip curvature measurement (B). In most experiments 10- × 1.5-mm interveinal strips or panels were prepared from growing leaves 8 to 10 cm long using double-bladed cutters. In this report leaf length refers to the distance from the distal tip to the base of the blade and does not include the petiole length. The strips were cut from the leaf, as indicated by the white rectangles, parallel to secondary veins radiating from the midrib so as to include a minimum of vascular tissues. Strips were prepared from the distal two-thirds of the leaf (middle and tip), with each leaf typically yielding six to eight strips. In one experiment, strips were prepared separately from each third of the leaf (base, middle, and tip) from leaves of various lengths (ages). B1, A typical strip immediately after excision viewed from the adaxial (dorsal) side; B2, an initial strip but viewed from the side. The degree of curvature (measured as shown from the angle created by the interception of the tangent to the two terminal portions of the strip) is indicated. B3, A typical strip and its measured curvature following a 20-h incubation without auxin; and B4, a typical strip and its degree of curvature following 20 h in 10 μM 1-NAA. B2-4, Abaxial surface on right.

The interveinal strips shown in Figure 1B, as well as those used in subsequent experiments, were cut parallel to the secondary veins radiating from the midrib to minimize the number of tertiary veins included in the strips (as shown in Fig. 1A). The epinasty induced by 10 μM 1-NAA did not seem to be limited to a growth response in the radial direction because, in a limited experiment, strips cut normal to the secondary veins (e.g. parallel to the midrib and across secondary veins) curved to 280 ± 46° ($n = 13$) over 20 h.

The dramatically increased epinasty induced by auxin in interveinal strips could have resulted from an auxin-induced suppression of elongation of the abaxial side and/or increased elongation of the adaxial side. Instead, it appears that auxin treatment increased the length of both leaf surfaces, with the adaxial surface increased more than the abaxial surface. Estimates of the length of the adaxial surface of auxin-treated strips (10 μM 1-NAA; 20 h) from digitized images indicated an increase in length (27.0 ± 4.1% of initial; $n = 12$). Assuming a leaf-strip thickness of 0.2 mm and using the measured curvature, we computed the length of the abaxial surface in the same strips to have increased (15.4 ± 2.8% of initial).

The auxin-induced growth of the adaxial and abaxial epidermises was presumably due to cell enlargement, because all cell division in the apical two-thirds of developing tobacco leaves has ceased by the time the leaf is 10-cm long (Poethig and Sussex, 1985). Cell expansion was apparently not limited to those tissues, because a microscopic examination of the palisade mesophyll of paired interveinal strips treated with and without auxin showed no indication that the cells of the auxin-treated mesophyll were separated by gaps or spaces. However, transects (0.4-mm long) through the palisade cells of the auxin-treated leaf fragments intercepted significantly fewer cells (16.7 ± 0.7) than were intercepted in the untreated tissue (21.0 ± 0.8), indicating that the auxin-treated cells were now larger than the untreated cells.

Ten micromolar 1-NAA was found to be at or close to the optimal concentration for curvature (Fig. 2). Figure 2 also shows that, although IAA was found to be an effective inducer of interveinal strip curvature, the strips were less sensitive to the naturally occurring auxin than to 1-NAA. The inactive auxin analog 2-NAA was found to be an ineffective curvature inducer, which is evidence that the epinastic response is specific to active auxins.

The ability of 1-NAA to induce epinastic curvature of excised midrib and secondary vein sections was also tested (Fig. 3). Both midrib and secondary vein tissues showed a similar maximal sensitivity to approximately 10 μM 1-NAA, as was found for interveinal tissues (Fig. 2). Secondary vein sections were found to curve more than midrib sections, but the maximum curvature response by both veinal tissues was much less than that found with interveinal strips (compare the ordinate scales in Fig. 2 with those in Fig. 3). The smaller response by vascular tissues was not a consequence of cutting away the mesophyll to within 0.5 mm of the vascular tissue because similar results were achieved in limited experiments in which 5-mm margins of interveinal tissues were retained to either side of the midrib (data not shown). Although veinal tissues were less epinastic in response to auxin than interveinal tissues, auxin treatment produced a nearly comparable increase in length. Midrib sections incubated for 20 h without auxin increased in length 10.4 ± 4.0% of initial ($n$ = 10), whereas those incubated in 10 μM 1-NAA were found to have elongated 21.0 ± 1.2% when pressed flat.

In a limited experiment in which leaves smaller than 8 cm were included with those used to prepare interveinal strips, some of the strips were found not to have an epinastic response to auxin. This observation of a possible developmental sensitivity to auxin in growing tobacco leaves was confirmed when the epinastic curvature in response to 10 μM 1-NAA was compared in interveinal strips prepared from a range of leaf sizes and from separate thirds along the length of the leaf (base, middle, tip) (Fig. 4). In the youngest leaves tested (those in which the leaf measured 4.5–6.0 cm in length), only the strips prepared from the distal one-third of the leaf (the tip) were epinastic in response to auxin treatment. In older leaves, first the middle (in 8- to 10-cm leaves) and then the basal thirds (in 12- to 14-cm leaves) were found to be sensitive to 1-NAA. Sensitivity was also found to decline progressively in older leaves, with first strips from the tip third (in 12- to 14-cm leaves) and then strips from the middle third of the leaf (in leaves 16- to 18-cm leaves) becoming less epinastic in response to 1-NAA. The largest mature, nongrowing leaves on the plants grown for these experiments were 18 to 24 cm in length.

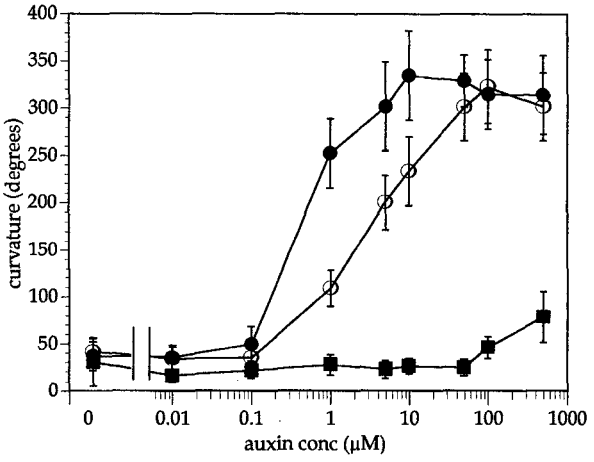


**Figure 2.** Effect of the auxins 1-NAA (●) and IAA (○) and of the inactive auxin analog 2-NAA (■) at a range of concentrations over 20 h on the epinastic curvature of interveinal strips. Interveinal strips (excised as described in Fig. 1A) were incubated in solutions containing 10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp (pH 6.0) plus the indicated concentration of either 1-NAA, IAA, or 2-NAA. The degrees of epinastic curvature were measured after 20 h as illustrated in Figure 1B. Errors represent 95% confidence limits; $n$ = 20 to 24.

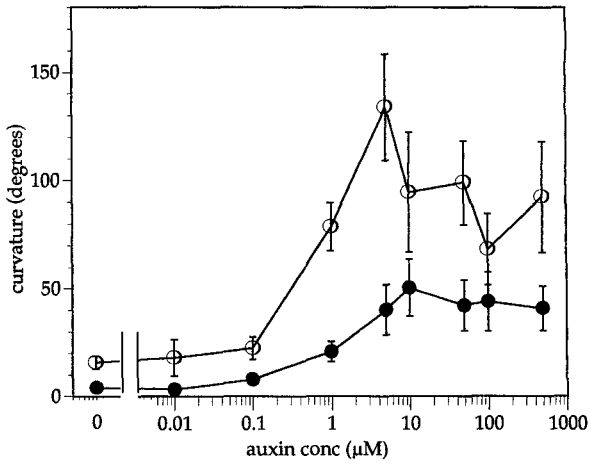


**Figure 3.** Effect of 20 h of auxin treatment on the epinastic curvature of midrib and secondary vein sections. Ten-millimeter sections from midribs (●) and secondary veins (○; which radiate from the midrib) were prepared from the middle third of leaves 8 to 10 cm in length after first cutting away the tissues on either side to within 0.5 mm of the vascular tissues. Segments were incubated for 20 h in solutions containing 10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp (pH 6.0) plus the indicated concentration of 1-NAA. Curvature was then determined as illustrated in Figure 1B. Errors representing 95% confidence limits are not shown when smaller than symbol size; $n$ = 24.

The time course of epinastic curvature induced in interveinal strips by auxin was also followed. Figure 5 shows that by 4 h a significant auxin-induced curvature was evident, and the curvature response continued to develop over many hours. The results in Figure 5 are not sufficiently resolved to make clear how early auxin-induced curvature was initiated, although a trend seems already evident by 2 h. Although the difference was not significant, repeatedly removing the strips from solution for measurement appeared to have a slight depressing effect on auxin-induced curvature over 24 h, as was evident from the slightly greater curvature by strips treated with auxin but only measured at the end of the experiment.

The possibility that epinastic curvature induced in tobacco leaf tissues may be an indirect result of auxin induction of ethylene production was tested through the use of AOA, a potent inhibitor of pyridoxal enzymes and thus of ethylene biosynthesis through inhibition of production of ACC, the immediate precursor of ethylene (Abeles et al., 1992). Figure 6 shows that AOA was found to be without effect on the curvature of strips in the absence of auxin over 20 h, but at 10 and 100 μM it significantly inhibited auxin-induced epinastic curvature. Furthermore, 100 μM AOA was more effective at inhibiting auxin-induced curvature if the strips were first preincubated in AOA for 2 h before

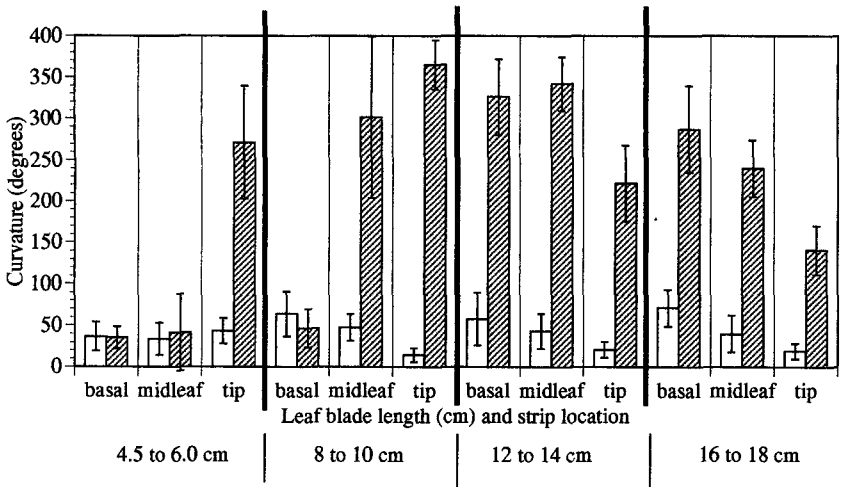


**Figure 4.** Effect of leaf age and strip position on the epinastic curvature induced in interveinal strips by auxin. Strips were prepared as shown in Figure 1A from the base, middle, and tip thirds of leaves in four size classes (4.5–6, 8–10, 12–14, and 16–18 cm). The strips were incubated for 20 h in either minus-auxin solution (10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp [pH 6.0]; open columns) or the same solution plus auxin (10 μM 1-NAA; striped columns), after which epinastic curvature was measured as illustrated in Figure 1B. Errors represent 95% confidence limits; $n = 16$.

inclusion of auxin in the treatment solution for a further 20 h. Such treatment inhibited more than half the curvature induced by auxin. In a limited experiment, another pyridoxal enzyme inhibitor known to inhibit ethylene production, aminoethoxyvinylglycine, was also found to partially inhibit auxin-induced epinasty.

The effectiveness of AOA inhibition of ethylene biosynthesis was tested by enclosing strips within 25-mL Erlenmeyer flasks containing 5 mL of solution and measuring the ethylene evolved after a 20-h incubation. As shown in Figure 7A, interveinal strips within enclosed containers retained an epinastic curvature response to auxin (10 μM 1-NAA) similar to that found with strips incubated in Petri dishes (Figs. 2 and 6) and was partially sensitive to 100 μM AOA (Fig. 6). Interveinal strips were found to evolve measurable ethylene over 20 h, and auxin was found to induce a nearly 4-fold increase in ethylene production (Fig. 7B). At 100 μM, AOA was found to reduce ethylene production by strips not incubated in auxin almost to unmeasurable levels and to reduce auxin-induced ethylene production to levels significantly less than those produced by strips not treated with auxin. These results show a poor correlation between ethylene biosynthesis and epinastic curvature and tend to suggest that AOA inhibition of epinastic curvature was unrelated to its inhibition of ethylene production.

As a further test of the role of ethylene in epinastic curvature by interveinal strips from tobacco leaves, we treated interveinal strips with ethylene by injecting

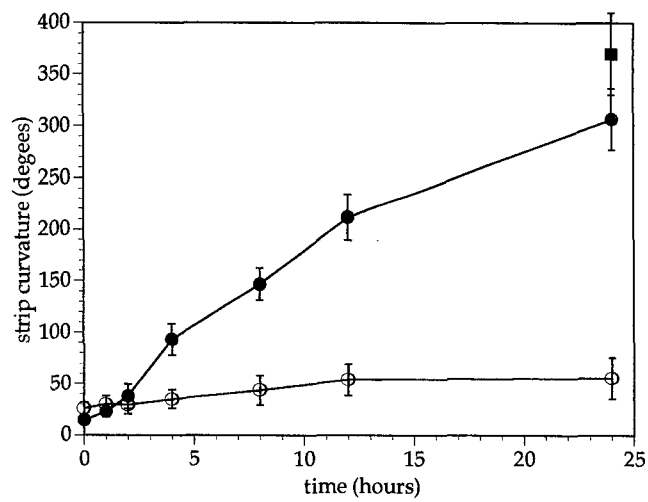


**Figure 5.** Time course of epinastic curvature of interveinal strips induced by auxin. Strips, prepared from the distal two-thirds of leaves 8 to 10 cm long, were incubated in either minus-auxin solution (10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp [pH 6.0]; ○) or the same solution plus auxin (10 μM 1-NAA; ●, ■). Strips were briefly removed from solution and the epinastic curvature was measured at the indicated times. The curvature of one set of strips incubated in auxin (■) was measured only at the end of the experiment. Errors represent 95% confidence limits; $n = 20$.

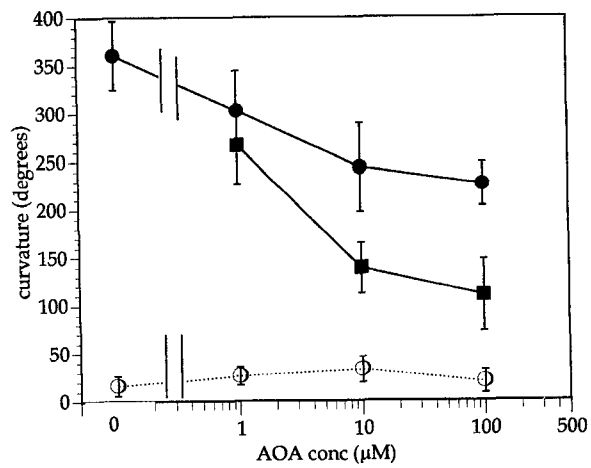


**Figure 6.** Effect of the ethylene production inhibitor AOA on the epinastic curvature induced by auxin in interveinal strips. Strips, prepared from the distal two-thirds of leaves 8 to 10 cm long, were incubated for 20 h in either minus-auxin solution (10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp [pH 6.0]; ○) or the same solution plus auxin (10 μM 1-NAA; ●) and, in each case, with 0, 1, 10, or 100 μM AOA as shown. Also shown is the effect on auxin-induced curvature of preincubating strips in the same concentration of AOA for 2 h before inclusion of 1-NAA in the medium for a further 20 h (■). Errors represent 95% confidence limits; $n = 16$.

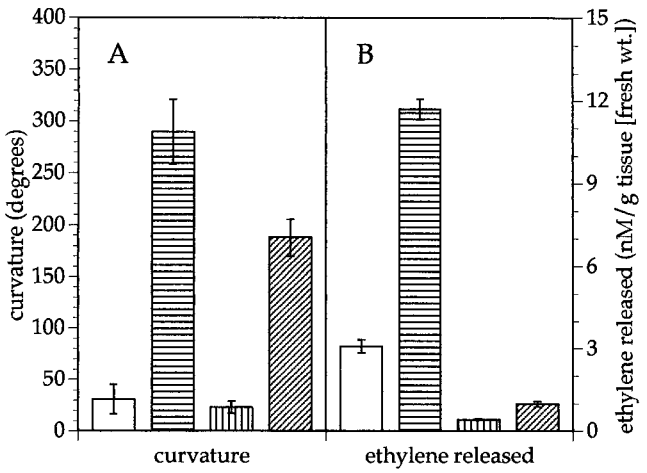


**Figure 7.** A, Effect of closed containers on the epinastic curvature of interveinal strips induced by auxin (10 μM 1-NAA) and its partial inhibition by AOA (100 μM). B, Effects of auxin and AOA on the biosynthesis of ethylene by interveinal strips in closed containers. Strips (40 per treatment), prepared from the distal two-third of leaves 8 to 10 cm long, were incubated for 20 h in minus-auxin solution (10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp [pH 6.0]; open columns), the same control solution plus auxin (horizontally striped columns), the control solution plus AOA (vertically striped columns), or the control solution plus auxin and AOA (diagonally striped columns). Incubation was in 25-mL Erlenmeyer flasks containing 5 mL of solution and closed with serum stoppers. After 20 h, ethylene production was determined with GC from 0.6-mL air samples taken from the flasks, after which the curvature of the enclosed strips was measured.

known quantities of the gas into stoppered 25-mL Erlenmeyer flasks containing strips in either control solution or in auxin (10 μM 1-NAA) and 100 μM AOA. These latter strips had first been preincubated in 100 μM AOA for 2 h. Figure 8 shows that ethylene administered at a wide range of concentrations did not rescue AOA inhibition of auxin-induced epinastic curvature. Furthermore, ethylene by itself was found to induce only a small but significant epinastic response and only when applied at 50 μL $L^{-1}$. These results indicate that auxin-induced curvature of interveinal strips from tobacco leaves was not a consequence of auxin-induced ethylene biosynthesis and confirms the conclusion that AOA-inhibited epinastic curvature is not a consequence of the inhibition of ethylene production.

## DISCUSSION

The development of the planar form of the mature tobacco leaf results from a surprisingly complex integration of the division and enlargement of cells in several tissues (Poethig and Sussex, 1985). Whereas plant hormones are presumably involved in the regulation of this process, auxin has generally been thought important within leaves only in the control of vein growth (Goodwin and Erwee, 1983; Digby and Firn, 1985; Hall and Langdale, 1996). A growth response by excised tissues in response to auxins does not, by itself, conclusively demonstrate a role for that hormone in the intact growth of those tissues. Nevertheless, the results presented here, that exogenous application of auxin can strongly induce epinasty of the interveinal tissues from young excised tobacco leaves, is evidence for a role for auxin as a regulator of the growth of both nonveinal and veinal tissues of tobacco leaves.

It is remarkable that the phenomenon of auxin-induced epinasty of nonveinal leaf tissues has so far gone unreported. One explanation is suggested by the developmental sensitivity to auxin (Fig. 4). Earlier tests of the effect of exogenous application of auxins to leaves may have been limited to very young or old tissues that were apparently unresponsive. Avery (1935), for example, reported that an auxin paste produced a growth response by the tobacco midrib but not the lamina as a whole when applied to very young attached leaves that were "still upright in the bud" and therefore likely to have blades even shorter than 4.5 cm. It is likely that the peak in epinastic sensitivity to auxin by midrib tissues precedes that of interveinal tissues because midrib sections treated for 20 h with 10 μM 1-NAA (Fig. 3) but excised from shorter leaves (e.g. <8 cm long) were somewhat more epinastic, curling 105 ± 19°.

Auxin-induced epinasty of nonveinal leaf tissues may not be a unique quality of tobacco, because transformants of petunia (*Petunia hybrida*) with high endogenous levels of IAA were reported to have epinastic leaves (Klee et al., 1987). The response may not be a uniform property of dicotyledonous angiosperms, however, which also might

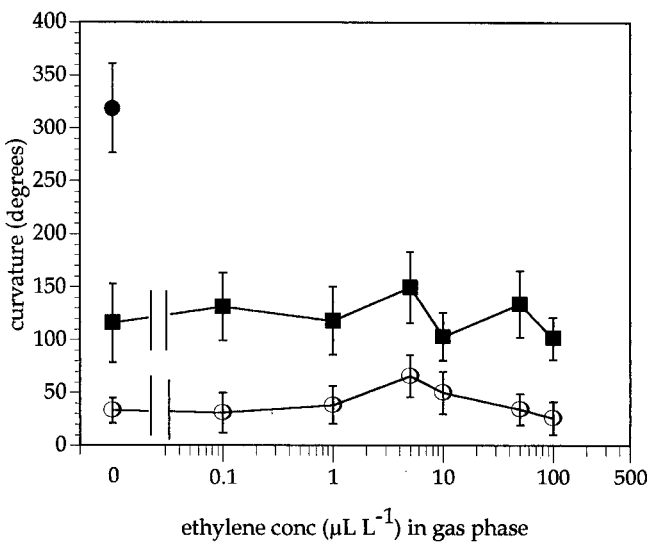


**Figure 8.** Effect of ethylene on both the epinastic curvature of interveinal strips and the inhibition of auxin-induced epinastic curvature by AOA. Curvature of strips, prepared from the distal two-thirds of leaves 8 to 10 cm long, was determined after they had been incubated for 20 h in control solution (10 mM Suc, 10 mM KCl, and 0.5 mM Mes/Btp [pH 6.0]; ○) or incubated with control plus AOA and auxin (10 μM 1-NAA, ■) and then pretreated with control solution plus 100 μM AOA for 2 h. Also shown is the curvature of strips incubated in control plus auxin and without ethylene treatment (●). Incubation was in 25-mL Erlenmeyer flasks containing 5 mL of solution and closed with serum stoppers, after which 0.6 mL of air was removed by syringe and replaced by an ethylene-containing injection appropriately concentrated to bring the ethylene concentration in the gas phase (μL $L^{-1}$) of the flask to the indicated levels.

help to explain why it has not been previously reported. Application of auxins to either young attached leaves or excised leaf discs from beans (*P. vulgaris*) resulted in a transient hyponasty (Lippincott and Lippincott, 1971; Hayes and Lippincott, 1976; Hayes, 1977). As in tobacco, auxin application resulted in an overall promotion of leaf growth, but, unlike tobacco, the growth promotion was relatively greater on the lower abaxial surface. The response in tobacco also differs in other ways from that found for bean leaves. In beans the hyponasty response is rapid and transient; maximum curvature was found within 4 h, and the lamina were uncurled again by 24 h. In tobacco, however, auxin-induced epinasty of leaf strips developed fairly steadily over 24 h (Fig. 5). The response then appeared to be near completion and nontransient, because strips left in solution for an additional 24 h also looked unchanged. In beans the vascular tissues were found to have a greater hyponastic response to auxin than did interveinal tissues, because leaf discs containing sections of the midrib or secondary veins showed greater curvature than discs free of major vasculature (Hayes and Lippincott, 1976).

Epinastic sensitivity to auxin appears transiently during the development of tobacco leaves and moves as a wave from the distal end of the blade to the base (Fig. 4). During tobacco leaf development, cell division followed by cell expansion ceases first in the distal portion of the blade and continues longest at the base (Avery, 1933; Poethig and Sussex, 1985). Curiously, the development of the epinastic sensitivity to auxin by excised leaf tissues, evident in Figure 4, appears to coincide with profound slowing of, if not complete cessation of, the growth rate in the intact leaf (Avery, 1933; Poethig and Sussex, 1985). Analysis of the auxin content of entire leaves suggests that IAA is continuously present in tobacco leaves, but the highest concentration on a fresh weight basis is in young leaves (Wightman, 1977; Sitbon et al., 1991). The leaves with the highest concentration of IAA, as well as two other auxins, phenylacetic acid and indolepropionic acid, were 6 to 9 cm in length and about to commence their 48 h of most rapid growth (Wightman, 1977). Our results, however, suggest that the contribution that the level of auxin makes to the regulation of growth in the intact tobacco leaf is likely to involve complex changes in concentration and sensitivity on a much smaller scale than between successively older leaves.

Numerous effects resulting from exogenous auxin application are known to involve induction of increased ethylene production (Abeles et al., 1992). This includes auxin-induced epinasty of tomato (*Lycopersicon esculentum*) leaf petioles, in which AOA has been shown to block the effect (Amrhein and Schneebeck, 1980). AOA had no effect on the tomato petiole epinasty induced by applications of ACC, indicating that the effect of AOA was through blockage of ethylene production. In other systems, however, the action of AOA may not be limited to the inhibition of ethylene production. For example, reduced root initiation in juvenile petioles of English ivy caused by aminoethoxyvinylglycine and AOA at 100 $\mu$M was not reversed by ethylene (Geneve et al., 1989). Our results showing a poor correlation between inhibition of ethylene biosynthesis by AOA and epinastic curvature (Fig. 7) suggest that the inhibition of auxin-induced epinasty by tobacco leaf tissues is also not a consequence of inhibition of ethylene production.

The inability of ethylene to reverse the inhibitory effects of AOA (Fig. 8) also supports that conclusion. Full biological activity by ethylene is at about 6.5 nM. Given its water/gas partition coefficient, this concentration would be in equilibrium with air containing 12 $\mu$L $L^{-1}$ ethylene (Abeles et al., 1992). However, we found no induction of epinasty by ethylene at 0.1, 1, 10, 50, or 100 $\mu$L $L^{-1}$ in the gas phase and only a very small effect at 5 $\mu$L $L^{-1}$.

In recent years two groups of investigators have published a number of reports in which tobacco leaves have been used as a model system for studying early events in the auxin growth response. As mentioned in the introduction, one group (Santoni et al., 1990, 1991; Masson et al., 1996) reported that the ATPase activity and proton pumping of plasma membranes from tobacco leaves are stimulated by auxin. The effect is relatively small, however, and dependent on the age and condition of the sampled leaves. In an important series of papers, other investigators (Ephritikhine et al., 1987; Barbier-Brygoo et al., 1989, 1991; Venis et al., 1990, 1992) used intracellular microelectrodes to characterize the response to auxin of the plasma membrane electropotential difference of protoplasts from tobacco leaf mesophyll. The major contribution of that work was to provide much of the evidence that auxin-binding protein 1 is in fact a bona fide hormone receptor (Jones, 1994; Napier, 1995; Venis, 1995). The results remain controversial, however, because of the relatively small membrane potentials reported (in the range of 0 to $-10$ mV). Other small values have been recorded for plant protoplasts (Pantoja and Willmer, 1986), but these are difficult to reconcile with the much more negative potentials generally found for intact cells (i.e. $\leq -100$ mV; Higinbotham, 1973). Recently, Van Duijn and Heimovaara-Dijkstra (1994) suggested that small values for plant protoplast membrane potentials are an artifact of microelectrode impalement and that an initial negative potential transient lasting a few milliseconds following impalement indicates that the membrane is much more polarized before impalement.

The results contained in the present report show that tobacco leaf tissues, including nonvascular tissues, are auxin-responsive and suggest that the tobacco leaf is an appropriate material for the study of auxin signal transduction. However, the developmental sensitivity of the auxin-induced epinasty evident in Figure 4 suggests that care should be taken in the selection of tissues for the preparation of subcellular or in vitro systems.

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